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Mohamed A.F. Noor
Louisiana State University

Katherine L. Grams
Louisiana State University

Lisa A. Bertucci
Louisiana State University

Yvette Almendarez
Louisiana State University

Jane Reiland
Louisiana State University

See next page for additional authors

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THE GENETICS OF REPRODUCTIVE ISOLATION AND THE POTENTIAL FOR GENE EXCHANGE BETWEEN *DROSOPHILA PSEUDOOBSCURA* AND *D. PERSIMILIS* VIA BACKCROSS HYBRID MALES

MOHAMED A. F. NOOR,[1] KATHERINE L. GRAMS, LISA A. BERTUCCI, YVETTE ALMENDAREZ, JANE REILAND, AND KELLEY R. SMITH

Department of Biological Sciences, Louisiana State University, Baton Rouge, Louisiana 70803

[1]*E-mail: mnoor@lsu.edu*

Abstract.—Hybrid male sterility, hybrid inviability, sexual isolation, and a hybrid male courtship dysfunction reproductively isolate *Drosophila pseudoobscura* and *D. persimilis.* Previous studies of the genetic bases of these isolating mechanisms have yielded only limited information about how much and what areas of the genome are susceptible to interspecies introgression. We have examined the genetic basis of these barriers to gene exchange in several thousand backcross hybrid male progeny of these species using 14 codominant molecular genetic markers spanning the five chromosomes of these species, focusing particularly on the autosomes. Hybrid male sterility, hybrid inviability, and the hybrid male courtship dysfunction were all associated with X-autosome interactions involving primarily the inverted regions on the left arm of the X-chromosome and the center of the second chromosome. Sexual isolation from *D. pseudoobscura* females was primarily associated with the left arm of the X-chromosome, although both the right arm and the center of the second chromosome also contributed to it. Sexual isolation from *D. persimilis* females was primarily associated with the second chromosome. The absence of isolating mechanisms being associated with many autosomal regions, including some large inverted regions that separate the strains, suggests that these phenotypes may not be caused by genes spread throughout the genome. We suggest that gene flow between these species via hybrid males may be possible at loci spread across much of the autosomes.

Key words.—*Drosophila,* hybrid sterility, introgression, reproductive isolation, speciation.

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Speciation has generally been defined as the evolution of barriers to gene exchange (isolating mechanisms) between taxa. Thus, over the past half-century, many studies of speciation genetics have focused on determining the genetic basis of these barriers to gene flow. Several general observations have emerged, particularly with regard to mating discrimination and hybrid sterility in *Drosophila* species. The X-chromosome has been suggested to have a disproportionate role in hybrid male sterility (Charlesworth et al. 1987; Coyne and Orr 1989), although more recent studies have questioned either the existence or the extent of this disproportionate role (Hollocher and Wu 1996; True et al. 1996). Both barriers generally appear to involve multiple genes, and few major gene effects have been identified (Wu and Palopoli 1994; Maside and Naveira 1996; Coyne and Orr 1998; Hollocher 1998; Ritchie and Phillips 1998; Ting et al. 1998). Often, almost every genetic marker used in a mapping study is significantly associated with the particular barrier being examined (e.g., Orr 1987; Coyne 1989). However, these studies and particularly those of mating discrimination have been limited in their scope because only a handful of genetic markers were used (see Coyne and Orr 1998). Thus, it has not been possible to determine whether these barriers are associated with genes spread across the genome or with localized regions of the genome.

An exciting area in the study of speciation is how, when reproductive isolation is not complete, certain parts of the genome can readily cross species boundaries while others cannot (e.g., see Wang et al. 1997; Butlin 1998; Rieseberg et al. 1999; Jiang et al. 2000). In hybridizing species, regions of the genome linked with barriers to gene exchange such as hybrid sterility or sexual isolation should penetrate poorly, whereas unlinked or loosely linked regions of the genome should penetrate more easily. If much of the genome is strongly associated with such barriers to gene exchange, then little interspecies introgression could ever occur. The low marker density of many previous studies makes it difficult to determine whether much or little of genomes are associated with such barriers and, therefore, the potential for interspecies introgression.

The genetic bases of various barriers to gene exchange have been investigated in *Drosophila pseudoobscura* and *D. persimilis* (Dobzhansky 1936; Tan 1946; Weisbrot 1963; Wu and Beckenbach 1983; Orr 1987, 1989; Noor 1997b). These species are isolated by F_1 hybrid male sterility, backcross hybrid inviability, strong species discrimination exercised by females, and an F_1 hybrid male courtship dysfunction. Nonetheless, they do hybridize at very low frequencies in nature because F_1 hybrids have been captured in the field (Dobzhansky 1973; Powell 1983), and introgression has been detected in one region of the fourth chromosome (Wang et al. 1997). These species have had a central role in studies of speciation, and the question of whether or how much gene exchange between them is possible has been debated (Dobzhansky 1973; Powell 1983, 1991; Kulathinal and Singh 2000; Noor et al. 2000a).

Previous genetic studies of the barriers to gene exchange that separate these species via males have focused primarily or exclusively on regions of the X-chromosome (see Table 1). Orr's (1987) study of hybrid sterility examined one marker per major autosome, and Noor's (1997b) studies of sexual isolation and hybrid male courtship dysfunction only examined one region of the third chromosome. Thus, much of the genome of these species has never been investigated with

TABLE 1. Summary of studies of reproductive isolation in hybrid males of *Drosophila pseudoobscura* and *D. persimilis.*

Study	Phenotype	No. X-chromosome regions tested	No. autosome regions tested	Effect detected
Dobzhansky (1936)	testis length	3	5	multiple X-linked factors suggested; large effect of chromosome 2, weaker effect of chromosome 3, weakest effect of chromosome 4
Weisbrot (1963)	inviability	1	3	significant effect of chromosome 3
Wu and Beckenbach (1983)	sterility	5	0	multiple X-linked factors suggested
Orr (1987, 1989)	sterility	2	3	multiple X-linked factors suggested; significant effects of each autosomal region, although barely significant for chromosome 4
Noor (1997b)	sexual isolation courtship dysfunction	2	1	multiple X-linked factors suggested no autosomal effect detected

regard to associations with barriers to gene exchange. Further, a recent power analysis of previous studies of sexual isolation suggested that Noor's (1997b) study (and many others) lacked sufficient power to detect potentially large effects of linked genomic regions due to the small sample size used ($n = 100$; Noor and Smith 2000).

Here, we expand on these previous studies to genetically map the four known barriers to gene exchange in backcross hybrid males of *D. pseudoobscura* and *D. persimilis*: hybrid male sterility, hybrid inviability, the hybrid male courtship dysfunction, and sexual isolation. Noor et al. (2000b) recently isolated numerous molecular markers (microsatellites and restriction fragment length polymorphisms [RFLPs]) from *D. pseudoobscura.* We evaluate the association between 14 molecular markers (12 on the autosomes: an increase of four to 12 times over recent studies) and the four barriers to gene exchange. Specifically, we seek to determine how much and what regions of the genome have the potential to introgress between these species via backcross hybrid males. We are also interested in the genetic basis of these barriers to gene exchange. To address these questions, we have concentrated on producing very large sample sizes rather than assaying many more genetic markers because we are interested in potentially weak effects associated with these genomic regions.

MATERIALS AND METHODS

Background, Crosses, and Handling of Flies

Drosophila pseudoobscura and *D. persimilis* have a metacentric X-chromosome and four telocentric autosomes. These species are separated by paracentric inversions on parts of four of their six chromosome arms spanning various lengths: six cytological bands on the left arm of the X-chromosome (XL), 11 cytological bands on the right arm of the X-chromosome (XR), five cytological bands in the center of the second chromosome, and various arrangements on the third chromosome (Tan 1935). However, recombination still occurs across the long, uninverted regions of these chromosomes (Sturtevant and Dobzhansky 1936; Dobzhansky and Sturtevant 1938; Noor and Smith 2000). Thus, genes affecting barriers to gene exchange can be identified across uninverted regions, but cannot be localized to particular locations within the inversions that separate these species.

Two strains were used in the backcross mapping experiment, *D. pseudoobscura* Flagstaff 1993 (third chromosome arrangement ''Arrowhead'') and *D. persimilis* Mount St. Helena 1993 (third chromosome arrangement ''Standard''). These chromosome arrangements differ by a single inversion along the middle of this chromosome spanning seven cytological bands, and they appear to be the most common within these species. Both strains have been maintained in the laboratory for several years and used previously in various other studies of mating discrimination (e.g., Noor 1995, 1997b). To identify strain-specific markers, we genotyped several individuals from numerous culture bottles at all surveyed marker loci.

Females from the *D. pseudoobscura* strain were crossed to males of the *D. persimilis* strain, and the resultant fertile F_1 females were backcrossed to males of each parental line. All crosses were carried out at 20 ± 1°C, 85% relative humidity, on standard sugar/yeast/agar medium. Backcross hybrid males were designated as BCps if they were offspring of *D. pseudoobscura* fathers or BCper if they were offspring of *D. persimilis* fathers. Bottles were cleared, and virgin backcross hybrid males and virgin pure species females were harvested 7 h later. The flies were then aged for 7 days in groups of five to 20 individuals. On day 7 after eclosion, males were separated into individual vials for 1 day to reduce crowding-mediated courtship inhibition (Noor 1997a).

On day 8, single females were aspirated into each vial containing a backcross hybrid male. Fly pairs were observed for 5 min after the onset of courtship or 5 min in total if no courtship occurred. Courtship was defined as wing vibration or attempted copulation by the male (Noor 1997b). Male courtship intensity and probability do not depend on the species of the female they are presented in these taxa (Noor 1996), so we have not separated the courtship data with regard to female species. If no courtship occurred, the fly was designated as a noncourter and removed. If courtship did occur, we recorded whether the fly was successful at copulating with the female for at least 30 sec during the observation period. Males that failed to achieve a 30-sec copulation were considered to be discriminated against by the female and were scored with a 0. Males that successfully copulated within their first two attempts were scored as 2. Other males that copulated within 5 min of courtship initiation but after two

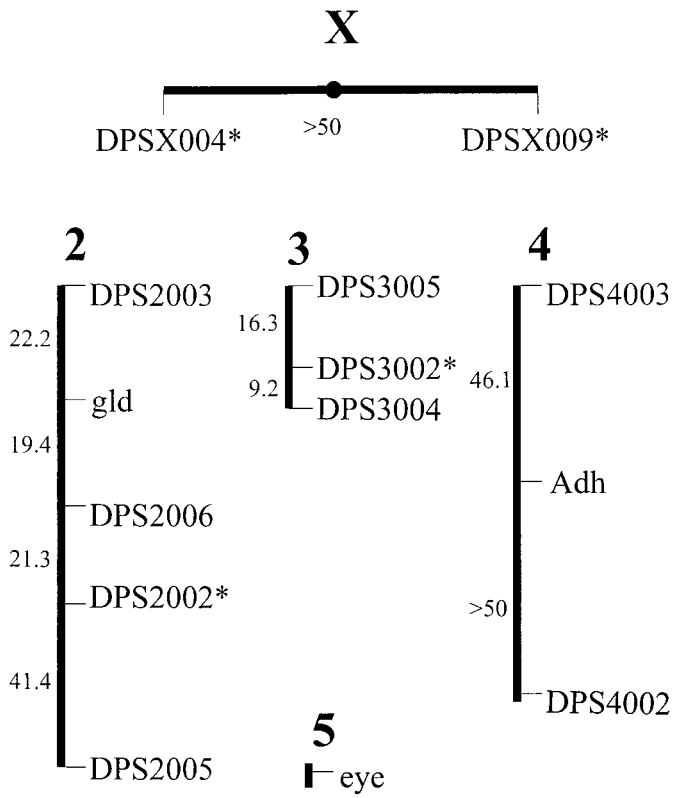

FIG. 1. Recombinational map (in Kosambi centimorgans) of molecular markers on the five chromosomes in backcross hybrids of *D. pseudoobscura* and *D. persimilis.* The centromeres of the autosomes are hypothesized to be at the ends closest to the top of the figure. *DPS4002* is unlinked to the other two fourth chromosome markers, and *DPSX004* and *DPSX009* are unlinked to each other. Markers indicated with an asterisk are linked to strain-specific inversions.

attempts were scored as 1. All observations were performed between 0700 and 1100 h, and each male and female were only used once.

Following courtship assays, male fertility was assayed by dissection of testes in insect Ringer's solution using the method of Coyne (1984). A male was scored as fertile (1) if he had any motile sperm and sterile (0) if no motile sperm were observed. Males were then frozen in labeled 0.6-ml microcentrifuge tubes. All methods were identical for assays of pure species flies except that no backcross was performed.

Molecular Markers

Most of the microsatellites and RFLPs used in this study have been described in detail elsewhere (Noor and Smith 2000; Noor et al. 2000b), including both their variability in natural populations and recombinational distances between markers in species hybrids (see also Fig. 1). In short, two markers were used on the X-chromosome, one on each chromosome arm and linked to the species-specific inversions: *DPSX004* and *DPSX009.* A previous study suggested that little recombination occurs along these arms outside of the inversions, although the chromosome arms recombine from one another freely (Noor and Smith 2000). Five markers were used along the second chromosome: *DPS2005, DPS2002* (linked to the inversion), *DPS2006, gld,* and *DPS2003.* The average spacing of these markers was approximately 25 cM. Three markers were used along the third chromosome: *DPS3005, DPS3002* (linked to the inversion), and *DPS3004,* which are spaced an average of 13 cM apart. Three markers were used along the fourth chromosome: *DPS4003* and *Adh,* which are approximately 46 cM apart, and *DPS4002,* which recombines freely from the other two markers. Finally, we developed a new RFLP marker in the *eyeless* gene on the nonrecombining fifth (dot) chromosome of this species. A 152-bp segment was amplified using primers 5′-ACTTCACAGGTTGTACAGTAATGTGTACC−3′ and 5′-GTAGGTCGAGGCTATGAGGTCG−3′. This product was then cut using *HinfI,* which produced a 100-bp fragment for the *D. pseudoobscura* allele and a 97-bp fragment for the *D. persimilis* allele.

The behavioral traits we are examining may have a low heritability (e.g., Williams et al. 2001), making an individual's phenotype a poor indicator of his genotype and necessitating our use of very large sample sizes. We also needed large sample sizes because the characters are often binary (mated vs. did not mate; Noor and Smith 2000) and because weak effects of particular genomic regions may also prevent gene exchange. Darvasi and Soller (1994) suggested that markers spaced every 20–30 cM give a high probability of quantitative trait locus (QTL) detection, although not necessarily precise position or effect. Thus, our coverage of the second, third, and fifth chromosomes is satisfactory for detection of associations with the barriers to gene exchange. The X-chromosome has already been studied extensively (Wu and Beckenbach 1983), so we have not focused on it here.

Microsatellites were assayed on agarose gels if the difference in size between the *D. pseudoobscura* and *D. persimilis* line alleles exceeded 10 bp or on acrylamide gels for smaller size differences. For microsatellite assays on acrylamide gels, one primer was ordered with an M13 tail at the 5′ end, and polymerase chain reaction (PCR) was performed in a 10-μl reaction volume with 0.5 picomoles of each primer, 0.4 picomoles fluorescent dye labeled M13, 200 μM dNTPs, 1 μl 10 × buffer (100 mM Tris pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 1 U Taq polymerase, and 1 μl from a 50-μl single fly squish prep (Gloor and Engels 1992). PCR was executed using a touchdown cycle (Palumbi 1996). Following PCR, 3 μl of LiCor (Lincoln, NE) stopping buffer was added to the reactions, and 1 μl of the PCR reaction was loaded onto an acrylamide gel (National Diagnostics Sequagel, Atlanta, GA) on a LiCor 4200 DNA sequencer for visualization. Individual males were scored as homozygous for a strain-specific allele or heterozygous for the alleles of the two strains. The *eyeless* RFLP was also assayed on acrylamide gels.

Microsatellite assays on agarose gels followed a similar PCR protocol except that primers were not labeled and 5 picomoles of each primer were used per reaction. Following PCR, 3 μl of Ficoll (Sigma Chemical Co., St. Louis, MO) loading dye was added to the reactions, and all 13 μl were loaded into a 2% agarose gel. The *Adh* RFLP was also assayed on agarose gels.

Allele Segregation Ratios

We evaluated the allelic segregation ratios at each of the markers used in this study. Alleles present the F_1 hybrid

TABLE 2. Fertility, courtship, and mating success of *Drosophila pseudoobscura*, *D. persimilis*, and their backcross hybrids. Numbers in parentheses indicate the sample size used for deriving the particular figure. See Methods for scoring details.

			Mated *D. pseudoobscura* females			Mated *D. persimilis* females		
Males	Fertile	Courted	Overall	Two attempts	Score	Overall	Two attempts	Score
D. persimilis	100% (137)	77% (298)	45% (110)	30%	0.755	84% (120)	78%	1.617
D. pseudoobscura	98% (164)	89% (245)	95% (112)	88%	1.830	54% (106)	30%	0.840
BCper	49% (1047)	83% (1282)	68% (512)	63%	1.307	68% (549)	59%	1.273
BCps	39% (1147)	70% (1482)	89% (503)	84%	1.730	55% (503)	41%	0.962

female should be found in the backcross male offspring at a frequency of 50%. Significant deviations from this expectation would suggest either hybrid inviability associated with a particular allele or meiotic drive altering the segregation ratio among the eggs that form the backcross hybrid males. As hybrid male inviability has been reported in these species (Weisbrot 1963), we presume this to be a more likely cause of such deviations from expectation (see Discussion for further support).

For each marker, we evaluated whether there was significant under- or overrepresentation of alleles at particular loci across backcross hybrid males using a chi-square test against an expectation of 50%. The two backcrosses were analyzed independently. Deviations reported as statistically significant had $\alpha < 0.05$ after a Bonferroni correction for 14 markers (Rice 1989), resulting in $\alpha < 0.003$ in practice.

Genetic Mapping

More than 2500 backcross hybrid males were genotyped for each marker: 1282 BCps males and 1482 BCper males (Table 2). For each trait, the two backcross directions were analyzed separately. Given that our data are binary rather than continuous and because of the spacing of our markers, standard programs for interval mapping or composite interval mapping could not localize putative QTLs within the intervals between our markers. We present our data in two ways. First, we present the raw effects of alternate alleles at each locus on the phenotypes being examined. For example, if 67% of males bearing one allele at a locus were fertile and only 2% of males bearing the alternate allele were fertile, we present these two numbers. This clearly illustrates the effects associated with each locus. Second, single marker regression analyses were performed using the QTL Cartographer (Basten et al. 1999) suite of programs (LRMapQTL, in particular). Using results from these analyses, we present both the proportion of the observed variation attributable to the marker (r^2) and its statistical significance following 1000 permutations. We acknowledge that estimates of effect are likely to be biased downward from single marker analyses due to recombination, but we present r^2 to illustrate approximate minimum magnitudes of effect. Associations reported as statistically significant had $\alpha < 0.01$. Only statistically significant associations are reported in the tables.

Although we have performed backcrosses in both directions, we cannot estimate additive and dominance effects associated with the markers we studied, as might have been possible with an F_2 cross. In the two backcrosses, no offspring were formed that are homozygous for *D. pseudoobscura* alleles at one locus but homozygous for *D. persimilis* alleles at another, which may have been possible if an F_2 cross could have been performed. If we were to combine the data from the two backcrosses and analyze them as an F_2 cross, we would overestimate effects associated with particular regions if any unlinked or loosely linked loci contributed to the phenotypes being studied. Thus, the two backcrosses must be analyzed separately.

For the mapping of the hybrid courtship dysfunction and hybrid male sterility, we also tested for weak QTLs and for interactions between X-chromosomal and autosomal regions using three methods. First, we used the CET protocol of Doerge and Churchill (1996) to identify potentially weak quantitative trait loci. When a strong effect was associated with a particular marker, the backcrosses were stratified into the separate classes for that marker and a reanalysis was performed with 1000 permutations. This procedure was repeated until no additional significant marker associations were detected. Second, we limited the dataset to only those backcross males bearing both X-chromosomal markers from the species that corresponds with the majority of the autosomal genotype (e.g., *D. persimilis* X-chromosome markers for BCper males). This stratification should make epistatic effects between X-chromosomal factors and heterozygous autosomal factors easier to detect. Finally, we performed multiple regressions of the phenotypes on X-chromosomal markers, autosomal markers, and their interactions (see Gurganus et al. 1999) to identify particular interacting regions. Because all of these procedures yielded essentially the same results, we report only the latter two.

No effects were detected on the fourth or fifth chromosomes for any phenotype, so these chromosomes are not presented in the tables or figures.

RESULTS

Pure Species Pairings

Drosophila pseudoobscura and *D. persimilis* males were nearly always fertile (Table 2). In addition, a large fraction of them generally courted any females they were presented, although the *D. persimilis* males (77%) were poorer than *D. pseudoobscura* males (89%) in that regard. Mating success was typically very high within species (84–95%) and substantially lower in interspecies pairings (45–54%).

Genetics of Courtship Dysfunction

As previously documented (Noor 1997b), many backcross males failed to court the female they were presented (Table

TABLE 3. Coefficients of determination (r^2) of associations between marker loci and phenotypes in backcross hybrid males of *Drosophila pseudoobscura* and *D. persimilis.* Only values statistically significant following Bonferroni correction are presented.

	DPSX004	*DPSX009*	*DPS2005*	*DPS2002*	*DPS2006*	*gld*	*DPS2003*	*DPS3004*	*DPS3002*	*DPS3005*
BCps										
Courtship	0.207	0.060	ns	0.006	ns	0.008	ns	ns	ns	ns
Sterility	0.430	0.014	0.007	0.050	0.032	ns	ns	ns	ns	ns
Mate *D. ps*	0.067	0.030	ns	ns	ns	ns	ns	ns	ns	ns
Mate *D. per*	0.019	ns	ns	0.026	ns	ns	ns	ns	ns	ns
BCps limited										
Sterility	—	—	0.028	0.363	0.141	0.019	ns	ns	ns	ns
BCper										
Courtship	0.026	ns	ns	ns	ns	ns	ns	ns	ns	ns
Sterility	0.274	0.015	ns	0.019	ns	ns	ns	ns	ns	ns
Mate *D. ps*	0.137	0.017	ns	0.036	ns	ns	ns	ns	ns	ns
Mate *D. per*	ns	ns	ns	0.047	ns	ns	ns	ns	ns	ns
BCper limited										
Courtship	—	—	ns	0.054	0.043	ns	ns	ns	ns	ns
Sterility	—	—	0.011	0.198	0.094	ns	ns	0.041	0.033	0.024

2). The fraction of BCps males that failed to court was significantly larger than the fractions of *D. persimilis* and *D. pseudoobscura* males that failed to court (Fisher's exact tests, $P = 0.0015$ and $P < 0.0001$, respectively). Noor (1997b) suggested that this phenotype may be a behavioral manifestation of Haldane's rule, as the heterogametic sex experiences a dysfunction only in hybrids. Because this dysfunction was manifested in F_1 hybrids bearing *D. persimilis* mothers, we predict that X-chromosomal loci from *D. persimilis* are interacting with either heterozygous *D. pseudoobscura* autosomal loci or the *D. pseudoobscura* Y-chromosome. If the X-chromosome interacts with the autosomes to produce this dysfunction, X-chromosomal effects should be observed in both backcrosses but should be most pronounced in BCps males because they are never homozygous for *D. persimilis* autosomal regions. In addition, autosomal effects should be detected in backcross males, and particularly in BCper males. In contrast, if X-Y chromosome interactions cause this dysfunction in hybrids, X-chromosomal effects should be detected only in BCps males and no autosomal effects should be noted.

We observed a strong effect associated with the X-chromosomal markers on courtship in males of both backcrosses, and particularly in BCps males (Tables 3, 4). This finding is consistent with the X-autosome interaction hypothesis for this courtship dysfunction. The marker *DPSX004* was associated with over 20% of the variance in this phenotype in BCps males, and the marker *DPSX009* was associated with an additional 6% ($P < 0.001$ in both cases). In both cases, bearing the *D. persimilis* allele reduced the probability of courtship in BCps males. Weak, although detectable, effects of autosomal loci were found in BCps males both *DPS2002* and *gld* ($P = 0.001$ and $P < 0.001$, respectively), each accounting for under 1% of the variance in the character. Bear-

TABLE 4. Effects of alternate alleles on backcross hybrid males of *Drosophila pseudoobscura* and *D. persimilis.* The first number in each entry is the value for males bearing only the homospecific allele (e.g., the *D. pseudoobscura* allele in BCps males) and the second is for males bearing one heterospecific allele. Mating percentages compare proportions that copulated within the first two attempts versus those that did not copulate within the first two attempts.

	DPSX004	*DPSX009*	*DPS2005*	*DPS2002*	*DPS2006*	*gld*	*DPS-2003*	*DPS3004*	*DPS3002*	*DPS3005*
BCps										
% Courting	85.4/42.2	78.9/56.0	ns	64.3/71.3	ns	63.7/72.1	ns	ns	ns	ns
% Fertile	66.6/1.7	33.3/44.9	43.2/35.0	50.6/28.8	48.1/30.7	ns	ns	ns	ns	ns
% Mate *D. ps*	89.2/69.8	88.7/77.2	ns	ns	ns	ns	ns	ns	ns	ns
% Mate *D. per*	35.9/57.1	ns	ns	33.0/46.0	ns	ns	ns	ns	ns	ns
BCps limited										
% Fertile	—	—	64.0/47.3	86.7/27.1	75.8/38.4	64.1/50.3	ns	ns	ns	ns
BCper										
% Courting	78.0/90.2	ns	ns	ns	ns	ns	ns	ns	ns	ns
% Fertile	69.7/16.3	54.7/42.5	ns	56.9/43.1	ns	ns	ns	ns	ns	ns
% Mate *D. ps*	45.5/79.8	57.5/68.2	ns	52.4/70.4	ns	ns	ns	ns	ns	ns
% Mate *D. per*	ns	ns	ns	71.2/49.3	ns	ns	ns	ns	ns	ns
BCper limited										
% Courting	—	—	ns	86.8/67.2	83.9/65.7	ns	ns	ns	ns	ns
% Fertile	—	—	80.3/71.1	93.5/54.7	88.1/61.4	ns	ns	83.6/66.0	80.8/64.8	80.1/66.4

ing the *D. persimilis* allele at these loci slightly increased the probability of courtship in BCps males. We did not initially detect any autosomal QTLs affecting courtship in BCper males.

If autosomal loci interact with the *D. persimilis* X-chromosome to produce the courtship dysfunction, these autosomal factors should be most detectable in BCper males when the X-chromosome is derived from *D. persimilis.* Thus, we limited our dataset to those individuals bearing both X-chromosomal markers (and therefore both inverted regions) from *D. persimilis* and surveyed the autosomes for QTLs affecting the courtship dysfunction in BCper males again. By doing this, we were able to detect a highly significant ($P < 0.001$) association on the second chromosome near *DPS2002* and *DPS2006*, which explained over 5% of the observed variation (Tables 3, 4).

To more precisely document that this courtship dysfunction results from an interaction between X-chromosomal loci and the region of *DPS2002*, we performed a multiple regression of courtship on *DPSX004*, *DPS2002*, and the interaction of these two regions (see Gurganus et al. 1999). We were able to identify both a significant main effect of the *DPSX004* region and a significant interaction between the two regions (*DPSX004*, $P < 0.0001$; *DPS2002*, $P = 0.43$; *DPSX004* × *DPS2002*, $P = 0.0003$) on whether BCper males courted.

Genetics of Hybrid Sterility

More than half of the backcross males surveyed did not possess any motile sperm (Table 2), a strong contrast to the high fertility of pure species males. As in previous studies (Dobzhansky 1936; Orr 1987), we found a very strong and highly significant effect of the left arm of the X-chromosome on this hybrid sterility, explaining 43.0% and 27.3% of the variation in hybrid male sterility in BCps and BCper males, respectively (Tables 3, 4). The right arm of the X-chromosome also contributed statistically significant but much weaker effects, explaining 1.4% and 1.5% of the variation in BCps and BCper males ($P < 0.001$ for all analyses). Counterintuitively, the *D. persimilis* allele at *DPSX009* enhanced fertility slightly in BCps males. This effect was still apparent when both *DPSX004* and *DPSX009* genotypes were considered together—the BCps males most likely to be fertile bore the *D. pseudoobscura* allele at *DPSX004* and the *D. persimilis* allele at *DPSX009.*

Again, an autosomal effect was detected in the vicinity of *DPS2002* in both backcrosses, accounting for 1–5% of the variation in each case ($P < 0.001$). No other autosomal regions appeared to independently contribute to hybrid sterility in our first analysis.

As above, we limited our analyses to just those individuals in the two backcrosses that bore homospecific X-chromosome arms (i.e., the X-chromosome arms borne by the father of the backcross males). This was done to reduce the variation resulting from the very large X-chromosomal effects observed in the two backcrosses. We did not survey males bearing the heterospecific X-chromosome arms because these males are almost invariably sterile. When we limited our dataset to males bearing both homospecific X-chromosome arms, the QTL(s) near *DPS2002* were more apparent, associated with 19.8% and 36.3% of the observed variation in BCper and BCps males, respectively (Tables 3, 4). The magnitude of the association in BCps males was especially strong: the difference in fertility between those bearing a *D. pseudoobscura* allele versus a *D. persimilis* allele at this locus was nearly 60%. A second QTL was also detected on the third chromosome near *DPS3004,* accounting for approximately 4% of this autosomal variation.

To more precisely identify epistatic interactions, we performed a multiple regression of fertility on *DPSX004*, *DPS2002*, *DPS3004*, and the interaction of X-chromosomal and autosomal regions (see Gurganus et al. 1999). By doing this, we were able to identify significant interactions between X-chromosomal and both autosomal regions on backcross male fertility (BCper: *DPSX004*, $P < 0.0001$; *DPS2002*, $P = 0.15$; *DPS3004*, $P = 0.14$; *DPSX004* × *DPS2002*, $P < 0.0001$; *DPSX004* × *DPS3004*, $P = 0.0041$; BCps: *DPSX004*, $P < 0.0001$; *DPS2002*, $P < 0.0001$; *DPS3004*, $P = 0.91$; *DPSX004* × *DPS2002*, $P < 0.0001$; *DPSX004* × *DPS3004*, $P = 0.51$).

Demographics of Hybrid Sterility

A recent study suggested that some hybrid males may develop motile sperm more slowly than pure species males (Maside et al. 1998), such that males scored as sterile at one age may be scored at fertile if they were assayed at a later time. Given that we have studied the genetics of sterility in hybrids of *D. pseudoobscura* and *D. persimilis*, we need to ensure that males that are scored as sterile at 8 days would not have been later scored as fertile. To this end, we surveyed the proportions of BCper males that possessed motile sperm at 11 days and 15 days after eclosion and compared these with the proportions that we surveyed at 8 days after eclosion. All surveyed males eclosed from the same media bottles.

We found no significant difference in sperm motility between males 8 days versus 11 days after eclosion (8 days, $n = 257$, 55.6% fertile; 11 days, $n = 229$, 52.0% fertile; $\chi^2 = 0.70$, $P = 0.4$). We also found no significant difference in sperm motility between males 8 days versus 15 days after eclosion (8 days, $n = 284$, 48.4% fertile; 15 days, $n = 260$, 52.9% fertile; $\chi^2 = 0.99$, $P = 0.3$). Paired comparisons using males that hatched on a particular day produced identical results. Fertility does not increase with increasing age in backcross hybrids of *D. pseudoobscura* and *D. persimilis* beyond 8 days after eclosion.

Genetics of Hybrid Inviability

Among BCps males, only two markers had segregation ratios significantly different from 50%: *DPSX004* (% *D. persimilis* allele: 40.8, $n = 1442$, $P < 0.001$) and *DPS2002* (% *D. persimilis* allele: 54.1, $n = 1439$, $P = 0.002$). Among BCper males, three markers deviated from expected segregation ratios significantly: *DPSX004* (% *D. persimilis* allele: 58.9, $n = 1221$, $P < 0.001$), *DPSX009* (% *D. persimilis* allele: 56.6, $n = 1209$, $P < 0.001$), and *DPS2002* (% *D. persimilis* allele: 43.4, $n = 1175$, $P < 0.001$).

Given that the segregation patterns of the X-chromosomal and autosomal loci are distorted in opposite directions, we also tested for interactions between these genomic regions

contributing to hybrid inviability. We used a chi-square test to compare the segregation ratio at the *DPS2002* locus when both *DPSX004* and *DPSX009* bore a *D. pseudoobscura* allele versus when the two X-chromosomal loci bore a *D. persimilis* allele. A significant chi-square value would suggest that loci in the region of *DPS2002* interact with loci in one or both of the X-chromosomal regions to cause the hybrid inviability found in the backcross males. Among BCps males bearing both X-chromosomal alleles from *D. pseudoobscura,* 232 bore the *D. persimilis* allele at *DPS2002* and 216 bore the *D. pseudoobscura* allele. Among BCps males bearing both X-chromosomal alleles from *D. persimilis,* 172 bore the *D. persimilis* allele at *DPS2002* and 128 bore the *D. pseudoobscura* allele. This difference was not statistically significant ($P = 0.15$). Among BCper males bearing both X-chromosomal alleles from *D. pseudoobscura,* 74 bore the *D. persimilis* allele at *DPS2002* and 132 bore the *D. pseudoobscura* allele. Among BCper males bearing both X-chromosomal alleles from *D. persimilis,* 190 bore the *D. persimilis* allele at *DPS2002* and 195 bore the *D. pseudoobscura* allele. This difference was statistically significant ($P = 0.0018$). The BCper genotype that was underrepresented bore a homozygous *D. persimilis DPS2002* genotype and a hemizygous *D. pseudoobscura* X-chromosome.

Genetics of Sexual Isolation and Mating Success

Drosophila pseudoobscura females

Both arms of the X-chromosome contributed to backcross male sexual isolation from *D. pseudoobscura* females (BCper: *DPSX004,* $P < 0.001$; *DPSX009,* $P = 0.003$; BCps: *DPSX004,* $P < 0.001$; *DPSX009,* $P < 0.001$; Tables 3, 4). Unlike the previous genetic study (Noor 1997b), we found a larger effect of the left arm of the X-chromosome than the right arm, particularly among BCper males. We detected a significant association of the region of *DPS2002* with mating success to *D. pseudoobscura* females in BCper males ($P < 0.001$), but not in BCps males ($P = 0.45$). Thus, the *D. pseudoobscura* autosomal factor(s) contributing to hybrid male mating success to females of that species appear to be dominant.

Drosophila persimilis females

The left arm of the X-chromosome was significantly associated with BCps male mating success to *D. persimilis* females ($P = 0.006$), but this region was not significantly associated with mating success in BCper males. The region of *DPS2002* had a significant effect on sexual isolation from *D. persimilis* females in both backcrosses (BCps, $P = 0.001$; Bcper, $P < 0.001$), each accounting for less than 5% of the observed variation.

Discussion

Using 14 microsatellite markers, we have genetically dissected four barriers to gene exchange in more than 2500 backcross hybrid males of *D. pseudoobscura* and *D. persimilis*: hybrid sterility, hybrid inviability sexual isolation, and hybrid courtship dysfunction. Three regions of the genome were strongly associated with all of these barriers to gene exchange: the left arm of the X-chromosome (XL), the right arm of the X-chromosome (XR), and the center of the second chromosome. The third chromosome contributed to hybrid sterility in one backcross, and the fourth and fifth chromosomes had no detectable association with any of the phenotypes examined. All detectable effects mapped onto the regions that showed inversion differences between these species (XL, XR, center of chromosomes 2 and 3), and the strongest effects were detected in regions that have inversion differences that are fixed within these species (XL and center of chromosome 2).

Our results suggest that much of the genome of *D. pseudoobscura* and *D. persimilis* may be able to introgress between species via male hybrids (excluding, of course, the completely sterile F_1 males). Regions that are inverted between these species are the least susceptible to introgression, particularly the XL and second chromosome inversions. However, much of the fourth and fifth chromosomes do not have strong associations with any barriers to gene exchange. Further, the most proximal region of the second chromosome (*DPS2003*; cytological location in Hamblin and Aquadro 1999) was also not associated with any barrier to gene exchange. Thus, these regions and particularly areas of the fourth and fifth chromosomes should have a high probability of being able to introgress between species when hybridization occurs. This suggestion is consistent with the results of Wang et al. (1997) suggesting that *Adh* (chromosome 4) has introgressed between these species.

Recombination is effectively inhibited in the areas of the genome that exhibit these inversion differences (Noor and Smith 2000), and single genes with large effects cannot be distinguished from numerous genes with smaller effects. Given that the strongest effects were associated with regions possessing fixed inversion differences, we cannot use standard crosses to further dissect their genetic bases. However, our results suggest that the phenotypes examined may not be both highly polygenic and homogeneously distributed throughout the genome. In particular, some large inverted regions did not possess any detectable effects on several phenotypes studied. If the phenotypes being studied were highly polygenic and distributed homogeneously across the genome, the inverted regions along the XR and third chromosomes should have been associated with at least 11% and 7% of the genetic variance, respectively. Given the large sample size used in this study, such differences would have had a high probability of detection. The absence of detected effects associated with such regions suggests either a limited number of genes contribute to these phenotypes or that the genes that contribute to them are clustered in particular genomic regions.

We do not interpret our observation of strong associations between reproductive isolation and the regions inverted between these species to be consistent with chromosomal models of speciation, because only paracentric inversions differentiate these strains. Additionally, F_1 and backcross hybrid males possess only one X-chromosome, and thus are not heterozygous for particular chromosomal arrangements. Finally, previous work has identified particular regions within some of these inversion differences that contribute to hybrid sterility, suggesting that the inversions themselves are not responsible (Wu and Beckenbach 1983).

It may be that regions of the genome close to the large effects we detected in the center of the second chromosome may still be able to introgress between these species. Concurrent work in another laboratory is now identifying patterns of introgression at these same genomic regions, and our combined projects will determine how much linkage to barriers to gene exchange is necessary to prevent gene exchange between *D. pseudoobscura* and *D. persimilis.* We discuss our current findings regarding the genetic basis of each of the four barriers to gene exchange in turn.

Hybrid Courtship Dysfunction

We mapped the genetic basis of the hybrid male failure to court (courtship dysfunction) to regions of the X- and second chromosomes. The left arm of the *D. persimilis* X-chromosome (XL) had the strongest effect on this phenotype, reducing hybrid male courtship propensity from 85% to 42% in BCps males. Our results suggest that genes on the XL interact with loci in or near the second chromosome inversion to produce this failure to court, although other loci may also be involved.

Hybrid behavioral dysfunctions are known in other species (e.g., Buckley 1969; Pashley and Martin 1987; Yoon 1991; Davies et al. 1997), but to our knowledge, this is the first study to identify the nature of the genetic interaction underlying it. In these cases, too, it appears to be restricted to the heterogametic sex, conforming to Haldane's rule (Haldane 1922). Like hybrid male sterility (e.g., Orr 1987; Johnson et al. 1992, 1993), the hybrid male courtship dysfunction in these species is caused at least in part by interactions between the X-chromosome and autosomes. Our observation that its genetic basis resembles that of hybrid sterility justifies claims that it is a behavioral manifestation of Haldane's rule.

We were initially unable to detect autosomal effects on hybrid courtship dysfunction in BCper males, but limiting our dataset to only those males bearing both *D. persimilis* X-chromosomes reduced the background variation such that autosomal effects could be identified. This procedure enhances the probability of detecting QTLs in backcross hybrid males when studying phenotypes caused by epistatic interactions between X- and autosomal loci. Such epistatic interactions appear to be major contributors to barriers to gene exchange, emphasizing the usefulness of this protocol.

Hybrid Sterility

We found that both arms of the X-chromosome contribute to hybrid male sterility, and interactions with autosomal factors are evident. In addition, we identified a very strong effect of the second chromosome inverted region contributing to the autosomal component of hybrid male sterility. The reduction in BCps male fertility associated with bearing one copy of the *D. persimilis* allele at *DPS2002* was striking (see Table 4). In contrast, loci on the third chromosome, which are also linked to a large inverted region, had a much smaller effect even though the third chromosome inversion (ST/AR) is somewhat larger than the second chromosome inversion (Tan 1935; Dobzhansky and Epling 1944). This contrast suggests either the presence of one or more genes with major effect or a disproportionate number of genes with small effects within the second chromosome inverted region contributing to hybrid male sterility.

Interestingly, we could find no other studies that have documented a specific interaction between an X-chromosomal region and one or more specific autosomal regions causing hybrid sterility, even though such interactions are often assumed to be a major cause of Haldane's rule (for review, see Johnson 2000). Whole chromosome analyses by Dobzhansky (1974) also implicated X-autosome interactions in hybrid sterility among subspecies of *D. pseudoobscura.* Other types of interactions are also sometimes implicated as causes of hybrid sterility (Johnson 2000). Orr (1987) identified a putative X-Y incompatibility contributing to sterility in male hybrids of *D. pseudoobscura* and *D. persimilis,* although he noted that ''autosomal genotype is not entirely irrelevant to fertility.'' Here, we have identified additional incompatible interactions contributing to hybrid sterility in this species pair.

Hybrid Inviability

We found alleles at three loci that appeared at frequencies deviating from expectation in adult backcross hybrid males. We inferred that such distortions would result from hybrid inviability rather than meiotic drive because inviability has been previously documented in these species (Weisbrot 1963). However, the distorted segregation switched directions between the two backcrosses. For example, the *D. persimilis* allele at *DPSX004* was significantly more prevalent than expected among BCper males, but significantly less prevalent than expected among BCps males. This switch strongly suggests that the distorted frequencies could not have resulted from meiotic drive because the hybrid female genotype that produced the two backcrosses was identical. We conclude that the observed adult allele frequency deviations result from hybrid inviability among backcross hybrid males.

Similar to results from studies of other taxa (Orr et al. 1997; Presgraves and Orr 1998; Orr 1999) and similar to our results on hybrid sterility, we detected an association between X-chromosomal and autosomal loci that contribute to hybrid inviability. In BCper males, hybrid inviability was associated with an interaction between a homozygous *D. persimilis DPS2002* genotype and a hemizygous *D. pseudoobscura* X-chromosome. Unlike other well-studied cases of hybrid inviability (e.g., Orr 1993; Coyne et al. 1998), the inviability we observe in this hybridization only appears in the backcross because it involves a homozygous autosomal segment from *D. persimilis,* whereas F_1 hybrids are heterozygous for their autosomes. Deleterious interactions involving homozygous autosomal segments of the genome in hybrids appear to be more common than deleterious interactions involving heterozygous autosomal segments (e.g., Breeuwer and Werren 1995; True et al. 1996) and may generally explain the phenomenon of hybrid breakdown in generations succeeding the F_1 generation.

Sexual Isolation

We found that both X-chromosomal and autosomal loci contribute to hybrid male sexual isolation from both *D. pseudoobscura* and *D. persimilis* females. With regard to *D. pseu-*

doobscura, the XL-chromosome arm contributed greatly to mating success and the XR-chromosome contributed a somewhat lesser, but still significant, effect. The second chromosome inverted region was also moderately associated with mating success to *D. pseudoobscura* females. With regard to *D. persimilis*, the XL and second arms contributed significant effects. Thus, the same general genomic regions appear to contribute to both premating and postmating isolation in these species, although it is unlikely that the same genes cause these effects.

In *D. pseudoobscura* and *D. persimilis*, sexual isolation results entirely from female species discrimination, so our study has addressed the genetic basis of the preferred male character (Merrell 1954; Noor 1996). A concurrent study has suggested that this preferred male character is their courtship song (Williams et al. 2001), and cuticular hydrocarbon differences do not appear to contribute to the observed sexual isolation (Noor and Coyne 1996). Our results concur with the genetics of courtship song differences in that numerous genes spread across the genome do not cause sexual isolation between these species.

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